

**STUDIES ON PROTEOLYTIC ENZYMES  
FROM  
MYXOCOCCUS VIRESCENS**

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MYXOCOCCUS VIRESCENS

Inaugural Dissertation

by

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Studies on proteolytic enzymes from *Myxococcus virescens*.

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This survey is based on the following papers, which will be referred to in the text by the given Roman numerals.

- I      Assay of elastase activity using trypsin as amplifying agent.  
Gnosselius, G. Anal. Biochem. 81:315-319 (1977)
- II     Purification and properties of an extracellular protease  
from Myxococcus virescens.  
Gnosselius, G. J. Bacteriol. In press (1978)
- III    Myxobacterial slime proteolytic activity  
Gnosselius, G. Arch. Microbiol. In press (1978)
- IV     Purification and quantification of three extracellular  
proteases from a strain of Myxococcus virescens exhibiting  
dispersed growth.  
Gnosselius, G. J. Bacteriol., submitted for publication  
(1978)

The myxobacteria are characterized by elongated, slender, non-flagellated vegetative, cells which have a gliding motility on the substrate. Many species form fruiting bodies which are sessile and stalked. The fruiting types secrete extracellular enzyme mixtures which have been shown to contain bacteriolytic enzymes attacking the cell-wall mucopeptide and which cause lysis of bacterial cells. The products obtained from this process are used as nutrients and the association of myxobacteria with dung is probably due to the large number of eubacteria contained in this material.

Although the presence of lytic enzymes among the products of the myxobacteria was reported as early as 1913 (1), more comprehensive investigations of these enzymes were only recently undertaken. The enzymes studied have mainly been those involved in the degradation of the cell-wall peptidoglycan. Attempts have been made to classify and characterize the bacteriolytic enzymes of different myxobacteria, including those derived from Myxococcus virescens. The myxobacterial proteases have thereby received attention only as regards their connection with the specific cleavage of the eubacterial peptidoglycan.

Extracellular proteases are produced by a wide variety of microorganisms (for a review see reference 2). Proteins must be broken down extracellularly by organisms using them as either sources of nitrogen or amino acids because protein molecules are too large to pass through the membrane of most microorganisms. The extracellular proteases cleave various, but by no means at all, proteins into peptides. The reaction seems to stop at this stage, but it has proceeded far enough to provide products whose size allow them to be assimilated.



Limited information has been published concerning myxobacterial proteases devoid of bacteriolytic activity. The results obtained have, in most cases, been obtained with crude, unfractionated preparations. The importance of these proteases for the myxobacterial cells was established by the observation that they are responsible for the lysis of heated or, in other ways modified, eubacterial cells (3, 4, 5). It is, however, still unclear just how the myxobacteria lyse the living eubacterial cell. This is especially the case with gram-negative bacteria, which demand close contact with a myxobacterium for lysis.

The investigation summarized in this survey treats the proteases present in the extracellular enzyme systems of Myxococcus virescens. Considerable effort was devoted to establish procedures for the fractionation of the crude systems and the purification of the individual proteases. Their action on synthetic substrates as well as their susceptibility to inhibitors have been studied. A more long-term aim has been to examine the properties of the proteases in order to further study the biological function of these enzymes.

#### Materials and Methods.

##### Organisms.

The strains of Myxococcus virescens used in this study originated from M. virescens B2 (6) and were derived by Ståhl (7). Strain S1H is

the best producer of extracellular lytic enzymes and it has been used earlier by Haskå (8). This strain does not grow dispersedly in liquid media. Strain D11 is a productive strain which grows stably in dispersion. It originates from strains examined by Haskå and Ståhl (9).

#### Cultivation.

The cultivation of M. virescens strain S1H (II, III) followed the method of Haskå (8). The cells were grown in rotary-shaken cultures with casitone medium (10). The cultivation of strain D11 in a fermenter (IV) was performed at constant temperature, pH and aeration. The stirrer speed was increased during growth. For continuous cultivation, fresh medium was furnished at a constant flow rate, which was calculated and adjusted to give steady-state conditions in the culture.

#### Enzyme assays.

The proteolytic enzyme activity was normally assayed as described by Haskå and Norén (11) with casein as substrate (standard method). With hemoglobin the assay was performed in 10% urea. In order to determine the inhibition of protein coagulation the standard method was modified (III). Proteolytic activity was then determined by the reduction of the viscosity of gelatin (12).

The determination of elastinolytic activity was a modification of the method developed by Naughton and Sanger (13), using trypsin as an amplifying agent (I).

Peptidase activity was determined by the release of ninhydrin-positive material (14) from synthetic peptides. Esterase activity was assayed according to Hummel (15)

Purified enzyme preparations, which were assayed for specific substrate activities, could be expressed as proteolytic enzyme units, as determined by the standard method (IV).

### Results and Discussion.

#### Cultivation.

Most fruiting myxobacteria will simply not grow as a homogenous suspension in liquid media, but grow rather in rings along the inside wall of the vessel or as clumps in the medium. This growth behaviour has caused difficulties in applying modern microbiological techniques to the investigations of these bacteria. Growth in a dispersed fashion has been attempted by changing the medium composition or by developing variant strains. Dispersed-growing strains of M. virescens have been isolated in this laboratory and the growth physiology studied by Haskå and Ståhl (9) and Ståhl (16). Generally, strains exhibiting dispersed growth are preferred when studying enzyme production in liquid media (7, 9, 17).

Myxococcus virescens, strain S1H, which grows in the clump-forming fashion, was cultivated in Erlenmeyer flasks on a rotary shaker (II, III). The techniques of Haskå (8) were followed when this strain was used for the separation of bacteriolytic enzymes from the bulk of the proteases. In order to obtain more well-defined conditions for growth, strain D11 was grown in a fermenter (IV). Two methods of growth determination were used to correlate the cell density with dry weight. With continuous cultivation, the dilution rate was calculated at each selected cell density, and then adjusted. Thus, neither the principle of the chemostate nor the turbidostat is strictly applicable to the continuous cultivation of myxobacteria. Such a density-dependent growth behaviour was suspected (9) and later confirmed (18).

#### Protease production.

Cell-free culture solutions from Myxococcus virescens contain proteolytic as well as bacteriolytic enzymes. These have been supposed to derive from intact myxobacterial cells, i.e. they are extracellular. No DNA has been found in the surrounding medium before the phase of decline (9, 11). Washed and disintegrated cells have not shown any proteolytic activity (11). For both the strains used in this study the proteolytic activity increased in proportion to the myxobacterial growth and reached its maximum before maximal growth was reached.

With M. virescens, strain S1H (II), the appearance of proteolytic activity in the culture medium was in accordance with the results given by Haskå (8). When strain D11 was grown in batch cultures (IV), the increase in proteolytic activity was considerably enhanced when the cell density exceeded 0.9 mg/ml. The extrapolated specific production rate corresponds to the initiation of this second production phase at a cell density of about 0.23 mg/ml. At this density a shift in the secretion of the individual enzymes may occur. The distribution of the three proteases analyzed was thus not uniform at different times of harvest. When continuously cultivated, cells at the higher density (1.0 mg/ml) produced proteolytic activity at a rate that was higher than that of the growth rate. At the lower density (0.3 mg/ml) the activity remained constant.

The strains of *M. virescens* used in this laboratory have been examined for growth, slime- and enzyme production. In many cases they have been extremely unstable and new variants have emerged with novel characteristics. The observed differences in growth behaviour between strain S1H and D11 can more or less be explained by differences in the nature of the slime (16). Slime production of strain D11 might also be connected with the protease production (IV).

Haskå (19) sometimes obtained at least two additional bacteriolytic enzymes from strain S1H. The protease production varied between the two strains used in the present study. From different variants of these strains, at least four additional proteases have been detected (unpublished results). Thus, the enzyme patterns obtained

do not appear to be directly connected with the growth fashion, e.g. whether the strain grows in clumps or dispersed.

#### Purification.

The purification procedures are summarized below.

The myxobacterial cultures were centrifuged and cell-free culture solutions were obtained. The enzyme solution was first concentrated by ultrafiltration without any appreciable loss in enzyme activity. However, a freezing-thawing procedure was found to be a prerequisite for high flow rates during ultrafiltration.

To the enzyme solution obtained from strain S1H was added concentrated potassium phosphate buffer, pH 7.8 (II, III). The precipitate was collected by centrifugation and subjected to a washing procedure involving repeated phosphate precipitation. The loss in proteolytic activity was 30% and almost 10-fold increase in specific activity was obtained with this purification step. Less successful attempts were made to purify the enzyme solution by fractional precipitation employing more classical methods.

The precipitate was dissolved in water and subjected to gel chromatography on Sepharose 6B. A high molecular weight protein-polysaccharide-lipid (PPL) complex was obtained in the void volume (III). The proteolytic activity appeared in the column volume of the column (II).

The proteases can not be adsorbed on a column of carboxymethyl-cellulose at pH 6.0 nor can most of the contaminating proteins (8).



Such proteins and part of the proteolytic activity were, however, adsorbed on diethylaminoethyl (DEAE)-cellulose at pH 7.8 and low ionic strength. By increasing the ionic strength, all the proteolytic activity was passed through a column of DEAE-cellulose and could then be adsorbed on such a column after a buffer exchange to pH 8.6. With gradient elution, the main part of the proteolytic activity emerged as a single component at about 40 mM  $(\text{NH}_4)_2\text{CO}_3$  and it consisted of about 50% of the applied activity. The recovery of the purified protease SII from the culture solution was 20% and a 500-fold purification was achieved.

The aim of the purification procedure with proteases from strain D11 (IV) was not only to separate the individual proteases from one another. At the same time the recovery should be high and the culture samples be of a convenient size. Fermenter batch cultures of 2 lit were harvested. The remaining content of the fermenter (0.5 l) could be used for reinoculation of a new batch. In this way the enzyme production during batch cultivation can be continuously examined.

After ultrafiltration, the proteolytic enzyme solution was further desalted on a Sephadex G-25 column. A second ultrafiltration reduced the volume to a size which made it suitable for preparative gel electrophoresis. By giving the sample lower conductivity than the buffer used, a narrow starting zone was obtained. This purification step utilized the fact that, after gel chromatography on Sephadex G-25, the proteases belonged to the low molecular weight components of the treated solution. By addition of Sepharose 6B on the polyacrylamide gel, high molecular weight substances with

relative mobilities of a magnitude corresponding to that of the proteases should be separated from the enzymes. The differences in relative mobilities of enzymes and colored low molecular weight impurities were also utilized. The ultimate result of the applied procedures was that small volumes devoid of interfering high molecular weight impurities could be analyzed. The recovery of the initial proteolytic activity in the culture solution was about 65%.

In general, when studying proteases, special care must be taken during the purification procedures, because of the risks for limited or mutual proteolysis. However, autodigestion was not found to be a significant problem during the purification of the proteases from M. virescens, if optimal stability conditions were maintained. Non-optimal conditions were thus avoided, for example during ion-exchange chromatography. The contact between the proteases from strain S1H and DEAE-cellulose was kept at a minimum in order to minimize the loss of proteolytic activity (II). Thus, ion-exchange chromatography was completely avoided with the proteases from strain D11 (IV). The high yield obtained with these enzymes is an indication that optimal stability was reached during their purification. The successful outcome of the phosphate precipitation step (II) required large amounts of proteinaceous and, probably, also slime material. Though the purification was satisfactory with this method, the recovery decreased when small culture volumes were purified. Preparative gel electrophoresis (IV) was found to be a convenient alternative. The buffer was continuously replaced in the electrode compartments in order to maintain optimal conditions.

The quantitative distribution of the purified enzymes could be illustrated most conveniently by gel chromatography on Sephadex G-100. Two of the proteases from strain D11 were eluted close to each other (IV). Separation by ion-exchange chromatography resulted in low recoveries and poor reproducibility. Analytical gel electrophoresis gave an excellent separation but low recoveries.

#### Criteria of purity.

Protease SII gave a single band on analytical polyacrylamide gel electrophoresis (II). Proteolytic activity corresponded to the stained protein band. The homogeneity of the protein was confirmed by SDS polyacrylamide gel electrophoresis. The proteases from strain D11 were purified as far as proteolytic homogeneity, i.e. the components were distinguished from each other by their specific substrate activities. Such homogeneity was confirmed by disc electrophoresis. The ratio for the specific activities (units per mg protein) for protease DI:DII:DII were of the magnitude 30:5:1. Isoelectric focusing of a solution containing the PPL complex revealed one sharp peak as measured by the ability to inhibit protein coagulation (III). Slime-bound proteolytic exhibited a specific activity towards gelatin corresponding to that of crystalline trypsin.

### Effects of inhibitors.

Several substances which do not take part in an enzyme reaction diminish its velocity. These substances are customarily known as inhibitors. A mere irreversible destruction of an enzyme, as, for example, denaturation by heat, strong acid, or other means, is not usually regarded as an inhibition. Many of these inhibitors act specifically as poisons of particular enzymes, and in some cases, in extremely small concentrations. Inhibitors which react specifically with amino-acid residues important for the enzymatic catalysis have been used to classify different proteases. The most studied extracellular proteases of microbial origin are serine proteases. These enzymes have in common specific and stoichiometric inactivation by certain organophosphorous compounds. Within this group there are two classes of proteases, the pancreatic serine proteases and the subtilisins, which, despite the homology of their active sites, exhibit completely different primary structures (20).

After incubation of the proteolytic enzymes from M. virescens with the agents tested, the remaining activities were assayed with casein as substrate. Protease SII was strongly inhibited by several metal ions (II). The results obtained with some other substances known to affect protease activities indicate that protease SII is inhibited by both chelating agents and substances which react with the reactive serine residues of proteases. Further attempts to obtain similarities to chymotrypsin and trypsin by studying the inhibition of the protease with chloromethyl ketones of tosyl-amino

acids and the soybean inhibitor yielded in a partly inhibited enzyme. Protease SII was also inactivated by ethylenediaminetetraacetate (EDTA). However, EDTA-inactivated enzyme could be reactivated by divalent cations of which calcium was the most efficient. Calcium ions are supposed to stabilize the structure of the enzyme protein. In the case of pancreatic elastase the effect caused by calcium may be related to a surface effect on the elastin substrate (I). Protease SII was thus classified as a serine protease (21).

All three proteases of strain D11 were inhibited by EDTA (IV). The effective concentration on protease DI and DII classifies them as metalloenzymes (21). Protease DIII was inhibited by higher concentrations of EDTA analogous to protease SII and also exhibited a reactivity towards diisopropylflourophosphate, which classifies protease DIII as a serine enzyme.

### Specificity.

A high specificity of an enzyme implies a strict limitation of the action of this enzyme to one substrate or to a very small number of closely related substances. The specificity of proteolytic enzymes was formerly related to the molecular size of the substrate and these enzymes were divided into proteinases and peptidases. However, the availability of a large number of synthetic peptides has shown that this distinction was incorrect, since proteinases were capable of hydrolysing very small peptides of suitable structure. The determining factor is not the size of the substrate molecule, but

the nature of the amino acid side-chains and other groups in the neighbourhood of the bond to be hydrolyzed. A given substance which is rapidly hydrolyzed by one protease may show little or no hydrolysis by another. Several substances have been proposed as specific substrates, whose hydrolysis is accomplished only by one particular enzyme, thus enabling that enzyme to be estimated in crude mixtures.

The extracellular proteases from M. virescens were usually assayed by the standard method with casein as substrate. The substrate specificities of the studied enzyme activities covered a relatively broad spectrum. Hydrolysis occurred with substrates typical for trypsin, chymotrypsin, papain, leucine-aminopeptidase, carboxpeptidase, elastase and collagenase.

The kinetics of protein hydrolysis, catalysed by the purified protease SII, revealed different  $K_m$ -values (II). The high value obtained with hemoglobin might reflect a limited susceptibility to proteolytic attack. Activity was obtained towards N-benzoyl-L-tyrosine ethyl ester (Bz-Tyr-OEt) and p-tosyl-L-arginine methyl ester, specific substrates for chymotrypsin and trypsin, respectively. The hydrolysis of other synthetic substrates as well as elastin also supports the conclusion that protease SII is a serine enzyme. Proteolytic enzymes exhibiting elastinolytic activity appear to be of interest both from a functional and an evolutionary point of view (c.f. reference 22). Elastinolytic and collagenolytic enzymes may contribute to the invasiveness of pathogenic bacteria. However, although protease SII exhibited



activity towards a specific collagenase substrate, it can not be considered a collagenase since native collagen was not attacked (23).

The peptide bond susceptible to protease SII seems to be formed by the carboxyl group of leucine and alanine. Alkaline proteases from various microorganisms appear to hydrolyze synthetic substrates, mainly at peptide bonds linking the carbonyl group of hydrophobic amino acid residues (24, 25). Morihara and Tsuzuki (26) have called these enzymes chymotryptic-like in their specificity, but considerable differences do exist. A selectivity for leucine has also been suggested as essential for the appearance of elastinolytic activity (27). In contrast to other serine proteases (26), L-leucine amide was hydrolyzed by protease SII though the amino group of leucine is unsubstituted. The  $\beta$ -naphthylamide was, however, not hydrolyzed like the pancreatic serine proteases. The insolubility and high spontaneous hydrolysis of the p-nitrophenyl ester might have concealed any reliable hydrolysis. The variety of peptide bonds cleaved by protease SII may be superficially interpreted, as indicating a low degree of specificity of the enzyme. However, the factors governing the specificity of protease SII can not be unequivocally discerned from examination of its relative action on synthetic substrates.

With elastin as substrate, a first order reaction was obtained with protease SII when trypsin was used as amplifying agent. This reaction behaviour indicates a high  $K_m$ -value relative to the substrate concentrations, a relationship quite contrary to that

of pancreatic elastase (I). Elastinolytic enzymes specifically attack the cross-linked regions of the elastin molecule. However, other regions in the protein can be hydrolyzed by these enzymes as well as by other proteases, i.e. trypsin. The differences in reaction order could be analyzed since only the specific elastinolytic activity was studied when trypsin was in excess. For protease SII the reaction order was found to be due to inability to adsorb on the substrate. Adsorption has been considered essential for elastinolysis (27).

The relative activities towards specific substrates could be used to distinguish the other two extracellular proteases produced by strain SIH from protease SII. The substrates tested on the proteases produced by strain D11 were chosen only on the basis of an "all-or-none" response of the enzymes (IV). From results on the hydrolysis of synthetic substrates, protease DI seems to have the most restricted substrate specificity. No obvious hydrolysis was obtained with any substrate that was not hydrolyzed by the other two enzymes, and protease DI also hydrolyzed the lowest number of substrates. Protease DII exhibited esterase activity against the chymotryptic substrate Bz-Tyr-OEt, although it seems to be a metalloprotease. Protease DIII hydrolyzed typical trypsin substrates, though a broader specificity was noticed. A peculiar characteristic trait of the enzyme towards benzoylarginine amide ( $\text{Bz-Arg-NH}_2$ ) was also exhibited. The substrate seemed to be cleaved at two sites; the first one was attacked before the second and in both cases amino groups were liberated (unpublished results).

To distinguish protease DIII and DII from each other, Bz-Arg-NH<sub>2</sub> and elastin, respectively, were used. The enzymes were fractionated on a column of Sephadex G-100 containing buffer and 0.1 M NaCl. Since elastinolytic activity is inhibited by high ionic strengths, the fractions had to be diluted. The high sensitivity of the trypsin amplifying assay (I) then made it possible to detect low levels of activity. The sensitivities of the specific assays with elastin and Bz-Arg-NH<sub>2</sub>, respectively, are about 15 and 25 times higher than that of the standard method. If the proteolytic activity, as determined by this method, is known, the activity of each enzyme in a mixture containing all three proteases can be determined.

The PPL complex (III) exhibited proteolytic activity against gelatin. Proteolytic activity only implies the hydrolysis of peptide bonds. At elevated temperatures in aqueous solutions, gelatin can be considered essentially devoid of secondary structure and the protein is then susceptible to almost all proteases. The specificity of the slime-bound proteolytic activity can not thus be deduced from its activity against gelatin.

#### Role of slime.

The ecological significance of a slime envelope is little known even for otherwise well-examined bacteria. Although the myxobacteria produce slime to a great extent, this property has attracted relatively little interest. The mechanism underlying the motility of

the myxobacteria may involve slime (29).. Vegetative cells cooperate in fruiting-body formation, which involves the embedding of myxospores in slime. Such an aggregation process indicates the action of a chemotactic substance (17). The slime of M. virescens might serve as a growth regulator (18) and influence the growth fashion (16).

Purified bacteriolytic enzymes from M. virescens are able to degrade certain gram-positive but no gram-negative bacteria (30). However, gram-negative bacteria are rapidly lysed in nature. This discrepancy can be overcome by sensitization with non-biological compounds; both detergent and heat treatment may denature the protein components in the outer membrane. However, fatty acids of the myxobacteria influence the viability of fungi (31) and are, in combination with bacteriolytic enzymes, able to lyse bacterial cells otherwise resistant to these enzymes (30). Furthermore, it is well known that close contact between the myxobacterium and its prey is necessary for lysis. The substance responsible for the initial alteration in the cell envelope has been suggested to be a lipase (39). The slime complex produced by M. virescens, strain S1H (III), seems to have a hydrophobic interaction with membrane lipids. Thus the slime may participate with a non-enzymic reaction in the initial attack on gram-negative bacteria.

After solubilization of the peptidoglycan by bacteriolytic enzymes, the cell contents of the eubacteria become accessible to other hydrolytic enzymes. The cell proteins are in their native

state and will probably not be attacked until they are denatured in some way. Loss of enzyme activity is a biological criterium for protein denaturation. Lysozyme was inactivated by the PPL complex and the process was accelerated by slime-bound proteolytic activity. The irreversible denaturing effect on hemoglobin resulted in an extremely good protein substrate for trypsin.

The denaturation of eubacterial cell proteins can be accomplished by the environment in a natural habitat, but the bacteriolytic capacity would be wasted if the myxobacteria could not themselves denature these compounds. Another function of the myxobacterial slime would thus be such a capability. After binding in the native state to the high-molecular-weight PPL complex, the proteins would be released in a denatured form. Such a mechanism would keep the proteins in the vicinity of the cell and facilitate the hydrolysis of proteins otherwise resistant to proteolytic attack.

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